INITIAL RESULTS ON USE OF SUBCRITICAL WATER FOR EXTRACTION AND FRACTIONATION OF ORGANIC AEROSOL FROM WOOD SMOKE AND DIESEL EXHAUST PARTICULATE AND APPLICATION OF TOXICITY TESTS

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ABSTRACT

In this study, subcritical water was used for the extraction and fractionation of organic atmospheric particulate. Two types of carbonaceous aerosol, diesel exhaust particulate (a relatively nonpolar matrix) and wood smoke particulate (a polar matrix), were sequentially extracted using subcritical water at temperatures ranging from 25°C to 300°C. The importance of each fraction was evaluated using two bacterial systems, the bacterial Polytox™ respiration test and the genotoxicity (SOS) Chromotest, and two mammalian systems, the *in vitro* cytotoxicity test and the hepatic mitochondrial respiration test. The results from the toxicity tests were related to the composition of individual fractions.

INTRODUCTION

The toxic effects of fine particulate aerosol (particles smaller than 2.5 μm) are discussed in a number of studies.[1,2] In contrast to inorganic aerosols, which are often well characterized, only 15–50% of the organic carbonaceous (OC) particulate mass has been characterized. The characterized compounds are almost exclusively nonpolar.[3] The limited knowledge on OC fractions is due to the use of organic solvents, which are able to extract only nonpolar or slightly polar organics. Moreover, with conventional solvent extracts, toxicity tests on OC are difficult to perform because the solvents employed are toxic for biological systems. Consequently, the toxicity of organic aerosols has been primarily studied for nonpolar OC (for example, polycyclic aromatic hydrocarbon [PAHs]), while the toxicity of significant polar fractions of OC is not known. These polar organics may also be important for mobilizing toxic metals from air particulates in biological systems by chelation reactions.[4]

Although not previously reported, subcritical water should have the ability to extract a broad range of polar to low-polarity OC from air particulate matter, as well as to provide extracts in a solvent (water), which is directly useful for toxicological tests. At lower temperatures (e.g., 50° to 100°C), water can extract polar compounds. With higher temperatures (e.g., to 250°C), the polarity of water decreases, and therefore, nonpolar compounds can be extracted.[5,6] Earlier studies have shown that compounds of different polarities such as phenols, PAHs, and alkanes could be sequentially extracted from a petroleum waste sludge by increasing subcritical water temperature.[7]

The toxicity of aerosol particulate is mostly evaluated indirectly through correlation of aerosol composition with epidemiological studies and/or inhalation studies requiring large amounts of samples directly delivered to tested organisms.[1,8,9] Because of faster evaluation (1-3 days) and low expenses, *in vitro* tests are often used for evaluation of different environmental matrixes such as soils or waste waters.[10] Several *in vitro* tests have been previously employed to express toxicity of aerosol particulate.[11,12,13]

In this work we have used subcritical water (from 25° to 300°C) to sequentially fractionate two carbonaceous aerosols; diesel exhaust particulate (a relatively nonpolar matrix) and wood smoke particulate (a polar matrix). To evaluate the importance of each fraction, four different toxicity tests were performed. Affects on respiration were examined using bacterial culture (Polytox™) and rat hepatic mitochondria. The SOS Chromotest was used to measure bacterial genotoxicity. Cytotoxicity to mammalian cells was analyzed using COS cells. The data obtained were related to composition of extracts, which was determined on the basis of carbon, hydrogen, nitrogen (CHN) and sulfur analysis, total organic carbon (TOC), gas chromatography/mass spectrometry (GC/MS) analysis, and diesel exhaust particulate analysis of metals.

EXPERIMENTAL

Sample material

Bulk diesel exhaust particulate was collected from the exhaust pipe of a diesel bus, homogenized and stored frozen until use. Bulk wood smoke particulate was collected from a chimney which vented an airtight wood stove burning a mix of hardwoods.[14]

Subcritical water extraction

Subcritical water extraction was performed in an apparatus previously described in detail.[15] The sequential extraction was performed at temperatures of 25°, 50°, 100°, 150°, 200°, 250°, and 300°C, holding each temperature for 30 min. The pressure was first held at 50 bar. At 250° and 300°C the pressure was increased to 100 – 150 bar to maintain the water in liquid state. The extracts were collected into 5 mL of water into a preweighted collection vial (40 mL). To prevent loss of samples, the collection vial was cooled in an ice bath. After each extraction, the system was washed with water heated from 25° to 325°C, and at 325°C with steam. The system was also washed in sequence with 5 mL of acetone, methylene chloride, and again acetone.

Toxicity tests

Each toxicity test was first performed on concentrated water extracts. Because the concentrated water extracts precipitated, additional tests were performed on dried samples, which were redissolved in dimethyl sulfoxide (DMSO). For each toxicity test, no toxic effect of DMSO was observed. The highest concentration of DMSO per assay was 1%. All tests were performed on the same fraction of water extracts.

Polytox™

The bacterial respiration test was performed with specialized bacterial cultures of Polytox™ (Interbio®, The Woodlands, Texas, USA). The freeze dried culture was grown overnight at 37°C mixed in buffered solution.[16] The culture was allowed to settle, then decanted and supernatant stored on ice and used for toxicity screening. The bacterial consumption of oxygen was measured with a biological oxygen monitor (YSI, Yellow Springs, Ohio, USA). The DMSO (10 µL) samples did not influence the oxygen consumption/formation.

Cytotoxicity test

The cytotoxicity test measured the inhibition of the viability of mammalian cells (COS) using MTT assay.[17,18] The cells were grown in tissue culture flasks (75 mL) in 12 mL of DMEM culture tissue media with 10% fetal calf serum and 1% penicillin streptomycin in a thermostated CO_2 incubator. When the confluence of the cells achieved 90–100%, the cells were removed from the flasks with 0.05% Trypsin and 0.53 mM EDTA•4Na, counted, and diluted to obtain a concentration of 10,000 cells per 180 µL of tissue culture media. The experiment was performed on 96-well microplates. On the first day, 180 µL of culture was introduced to each well, and on the second day 10 µL of sample diluted in tissue culture media was added, so the final concentration of DMSO in tissue culture (if used) was <1%. All samples were studied in quadruplicate with two blank controls and were incubated overnight. The toxicity was evaluated using MTT assay.[17,18] The results were analyzed at absorbance of 570 nm using a ELISA plate reader (Spectromax plus 384, Molecular Devices).

Before the toxicity test, the pH of all samples was adjusted with 1M NaOH to 7.

SOS Chromotest

SOS Chromotest is a bacterial genotoxicity test developed by Quilllardet et al.[19] as an alternative to the Ames test[20].

An *Escherichia coli* strain PQ 37 (obtained from Pasteur Institute, France) containing a fusion gene of a ß-galactosidase (ß-Gal) gene (lacZ) with an SOS response gene (sfiA) was used in this assay. Activation of the SOS repair system by genotoxic agents is measured by photometric determination of the ß-Gal enzyme activity. The procedure for the miniaturized test was based on previously published procedures.[21,22,23] A sample of overnight culture was diluted with 10 mL of LA medium[20] and incubated at 37°C in a gyratory incubator until it

reached optical density of OD_{600}= 0.5, then 1 mL of culture was diluted with 9 mL of L medium[20]. For metabolically activated experiments, activity of bacteria used was 4x higher.[24] The tester strain (100 µL) and 10 µL of samples were introduced into 2 mL polypropylene tubes, which were incubated with shaking for 2 hr at 37°C in a water bath. For the toxicity determination of each extract, 8 tubes were prepared, two with L medium instead of the culture (blanks) and 6 with the culture. Half of the samples (blank + 3) were used for the determination of alkaline phosphatase (AP) activity and the other half for determination of ß-Gal activity.

ß-Gal assay was based on Miller's procedure[23]. After incubation of the sample with the culture, 0.9 mL of ß-Gal buffer, 30 µL 0.1% SDS, and 50 µL chloroform were added, mixed, and incubated at 37°C for 5 min, than 200 µL of 0.4% o-nitrophenyl-ß-D-galactopyranoside (ONPG) (Aldrich, USA) was added and the mixtures were incubated for 25 – 60 min (time sufficient for color development) with shaking at 37°C in a water bath. The conversion of ONPG was stopped by 200 µL of 4 M $NaNO_3$. The procedure for AP determination was similar to ß-Gal assay, only instead of the ß-Gal buffer, an AP buffer was used, and instead of ONPG, p-nitrophenyl phosphate disodium salt hexahydrate (PNPP) (Aldrich, USA) was used. The reaction was stopped after 10 min with 200 µL of 6 M NaOH. The extent of reaction was measured on a Spectrometer 65 DU (Beckman, USA) at absorbance of 405 nm against a blank (tube containing all ingredients but bacteria). A similar procedure was followed for metabolically activated experiments, with rat liver mixture S9 (In vitro Technologies, Maryland, USA) added to the culture prior to the incubation with extracts.[21,24]

Similarly as for cytotoxicity tests, the pH of samples was adjusted with 1M NaOH.

Mitochondrial respiration assay

Liver were isolated from adult, male, Sprague-Dawley rats (Harlan) using a modified procedure of Picklo and Montine.[25] Isolation buffer consisted of mannitol (0.21 M), sucrose (70 mM), HEPES (5 mM), EGTA (1 mM), and bovine serum albumin (BSA 1 mg/mL) with a final pH of 7.4 at 4°C. Rats were deeply anesthetized and the liver removed and homogenized in a 10-fold excess (v/w) of buffer. Homogenates were centrifuged at 3000 g for 2 minutes (4°C) to pellet nuclei and cellular debris. The supernatant was then centrifuged at 12000 g (4°C) for 10 minutes. The pellet was resuspended in 10 mL of homogenization buffer and centrifuged at 12000 g (4°C) for 10 minutes. The pellet was resuspended in buffer (10 ml) without BSA and was centrifuged at 12000 g (4°C) for 10 minutes. This final pellet was resuspended in 0.5 mL of buffer without EGTA. Protein concentration was measured using protein assay reagent (BioRad) with BSA as the standard.

Respiration was measured using an oxygen electrode (YSI International) and oxygen consumption chamber (Gilson Medical Electronics).[25] Experiments were performed at 37°C. Assay media contained 125 mM KCl, 5 mM $MgCl_2$, 2 mM KPO_4, and 5 mM HEPES-KOH (pH 7.4). Complex I-linked substrate (5-mM glutamate) was used to stimulate State 4 respiration. State 3 respiration was measured for 2 minutes following ADP (1 mM) addition.

Elemental analysis

CHN analyses were performed on wood smoke and diesel exhaust particulate water extracts of 500 mg and 200 mg, respectively. The extracts were dried under a stream of nitrogen and submitted to analysis. Total organic carbon analyses were performed directly on water extracts obtained by extraction of 100 mg of particulate using Environmental Protection Agency (EPA) method 415.1. To obtain general information on total composition of wood smoke and diesel exhaust particulate X-ray fluorescence spectrometry semiquantitative scans were performed on both particulate samples. Diesel exhaust particulate, contained higher quantities of metals. Therefore, the extracts were analyzed using inductively coupled plasma mass spectrometry (ICP/MS) for selected metals.

Gas chromatographic analysis

Prior to analysis, the water extracts were dried and redissolved in 200 μL of acetone. GC/MS analyses were performed using a Hewlett-Packard model 5890 GC with a Hewlett-Packard model 5972 MS in the full-scan mode (45-500 *m/z*) with electron impact ionization. Tentative quantifications were performed on gas chromatograph with flame ionization detection (GC/FID) on a Hewlett Packard model 5890 Series II gas chromatograph equipped with an autosampler. Quantitative analyses were based on the external calibration with compounds typical for diesel exhaust and wood smoke particulate such as polyaromatic hydrocarbons, alkanes, syringol, and quaiacol derivatives. On both instruments chromatographic separations were accomplished with a 30 m DB-5 column with a 0.25 mm I.D. and a 0.25 μm film thickness (J&W Scientific, Rancho Cordova, CA, USA) with injections in the splitless mode. The oven temperature was held at 40°C for 0.2 min followed by 10°C/min gradient to 320°C and then held for 20 min.

RESULTS AND DISCUSSION

Two common carbonaceous aerosols, diesel exhaust particulate (relatively nonpolar matrix) and wood smoke particulate (polar matrix) were sequentially extracted using a range of subcritical water temperatures from 25° to 300°C. The toxicological importance of individual fractions was studied on four separate models, bacterial and mammalian cell respiration, mammalian cell cytotoxicity, and bacterial genotoxicity. Each test was first performed using concentrated water extracts, but because the cooled concentrated water extracts precipitated, the tests were also performed on dried extracts, which were dissolved in DMSO. Both tests gave similar trends, but the response was more pronounced in DMSO dissolved extracts. Therefore, all data presented are based on the use of DMSO.

Toxicity of wood smoke particulate

The toxicity tests were performed on the same proportion of each temperature extract. This approach allowed comparison of the contribution of each water fraction to the total toxicity of whole sample. Results obtained with different toxicity tests are obviously specific to different classes of compounds. The data on Polytox^{TM} show low sensitivity of this test (Figure 1a). The tests on extracts dissolved in water, which were performed with larger

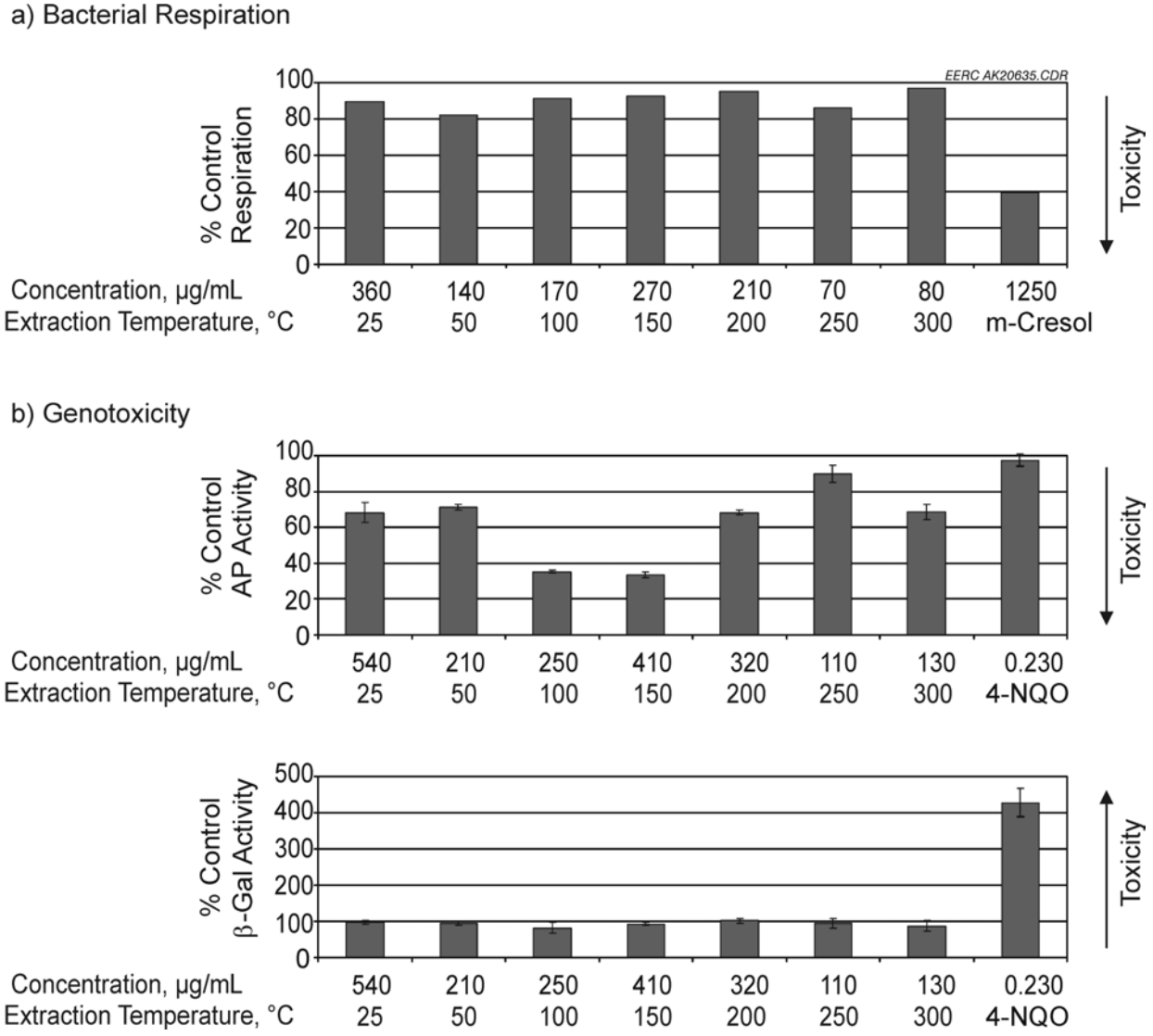

Figure 1. The results from bacterial toxicity tests performed on wood smoke particulate fractions obtained sequentially at temperatures 25°–300°C. a) Polytox^{TM} 5% of each extract, b) Genotoxicity (0.5%).

fractions of samples, (20% and 40%) did show inhibition to 20% of normal bacterial respiration of 25° and 50°C fractions (polar). For the second bacterial test, the SOS chromotest (Figure 1 b), two sets of data were obtained: (1) The alkaline phosphatase activity representing the total protein synthesis, which shows that fractions collected at 100° and 150°C have the highest toxicity; and (2) the genotoxic response, which is expressed as a formation of β-galactosidase enzyme, for wood particulate extracts no genotoxic response was observed. It is known that many compounds such as PAHs are genotoxic after enzymatic metabolic activation, when arylhydroxylase in liver metabolizes PAHs into hydroxylated compounds, which are soluble and toxic. Therefore, tests were also performed with rat liver microsomal S9; however, no increase in genotoxicity was observed after activation.

Each test was validated on test compounds, m-cresol for the Polytox™ and 4-nitroquinoline oxide (NQO) for the genotoxicity test. Similarly to Polytox™, the cytotoxicity test using mammalian COS cells (Figure 2a) showed higher toxicity of more polar fractions, however, it is important to note that the extracted mass was higher in polar fractions.

The results from the test on rat liver mitochondrial respiration (Figure 2b) show uncoupling activity of higher temperature extracts 200°–300°C (state 4). In addition to it, the extracts of 150° and 200°C blocked ADP stimulated respiration (state 3).

Toxicity of diesel exhaust particulate

The toxicity of diesel exhaust particulate water fractions is shown in Figures 3 and 4. Both the water fraction obtained at 25°C (polar materials), which was dried and dissolved in DMSO, and the concentrated water showed significant inhibition of bacterial respiration (Figure 3a). In contrast to wood smoke particulate, significant genotoxicity was observed for fractions extracted between 100°C and 250°C. For those temperatures the protein synthesis was also inhibited (Figure 3b).

The cytotoxicity of diesel exhaust (nonpolar matrix) showed increasing toxicity with increasing extraction temperature. This trend is opposite to (Figure 4a) the cytoxicity of wood smoke particulate (polar matrix) fractions (Figure 2a) and may correspond with the difference of sample polarities.

Mitochondrial respiration significantly increased for the fractions collected above 100°C, this may correspond to the extracts uncoupling activity (state 4). The same fractions had also blocked state 3 respiration. Interestingly, the 25°C fraction for which the inhibition of bacterial respiration was observed did not show uncoupling activity but did block ADP stimulated respiration (state 3).

Characterization and possible relationship to toxicity results

Detailed characterization is needed to explain the toxicity of different fractions. Therefore, elemental analyses for CHN, and S were performed, the total organic carbon was determined, the extracts were analyzed using GC/MS, and diesel exhaust particulate was characterized for metals.

Elemental analysis

The mass extracted, CHN, and sulfur contents for both particulate matters are shown in Figure 5. The gravimetric data show the most polar water (25°C) extracted the highest mass of particulate. The total extracted particulate with subcritical water extraction (25°–300°C) was 42 wt. % and 34 wt. % for wood smoke particulate and diesel exhaust particulate, respectively. Figure 5 shows that CHN and S composition corresponds to approximately

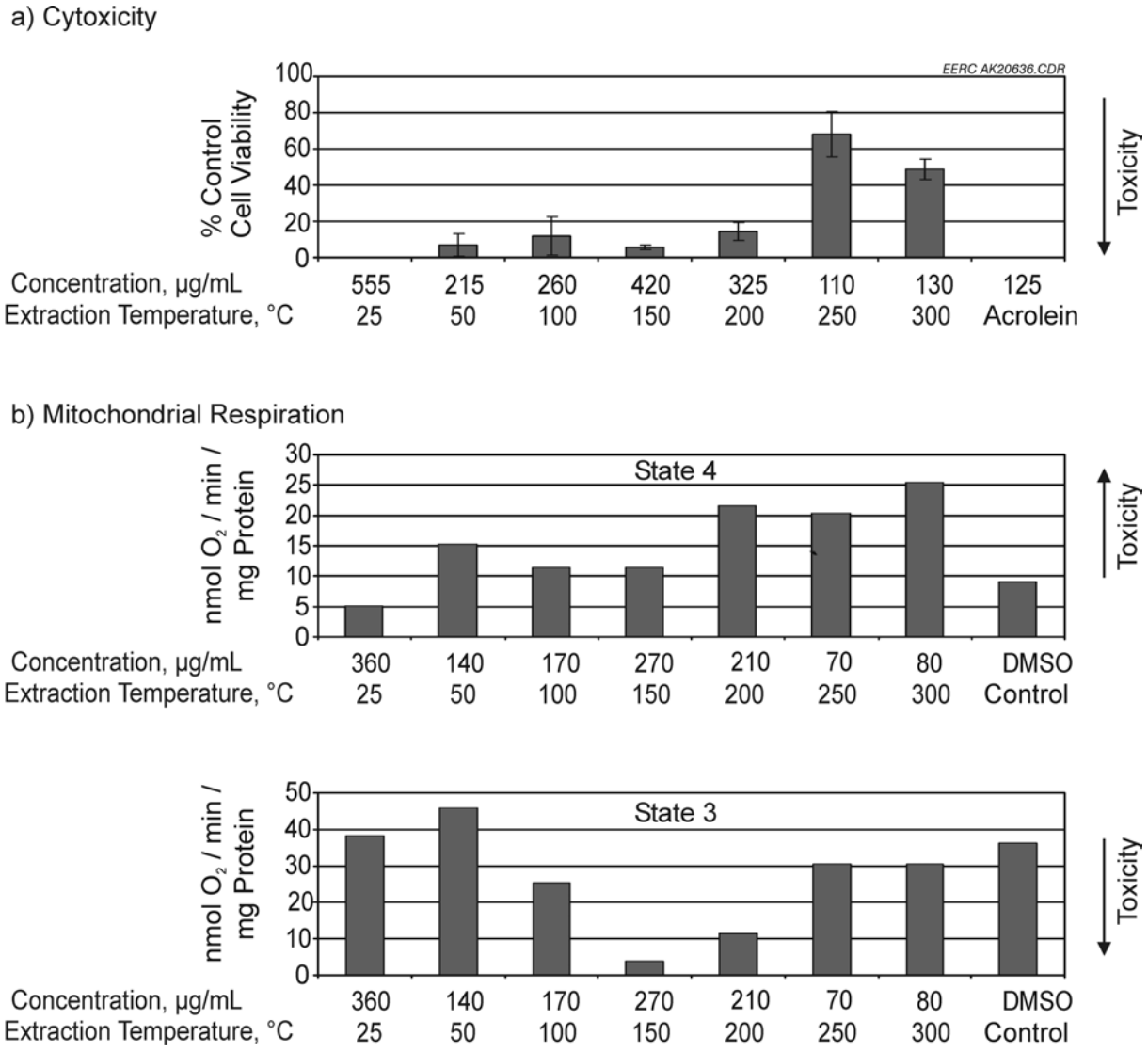

Figure 2. The results from toxicity tests performed on wood smoke particulate fractions obtained sequentially at temperatures 25°–300°C. a) Cytoxicity 1% of each extract compared to acrolein b) Mitochondrial respiration (5%)compared to effect of DMSO.

40–60 wt. % of the extracted mass for each fraction. To determine if the carbon extracted with water is mainly organic carbon (as opposed to carbonate or graphitic carbon), elemental carbon (total carbon) values were compared to total organic carbon (TOC) values in the water extracts (Figure 6). Good agreement between elemental carbon and TOC values demonstrates extracted carbon is essentially all organic. Organic carbon determinations can

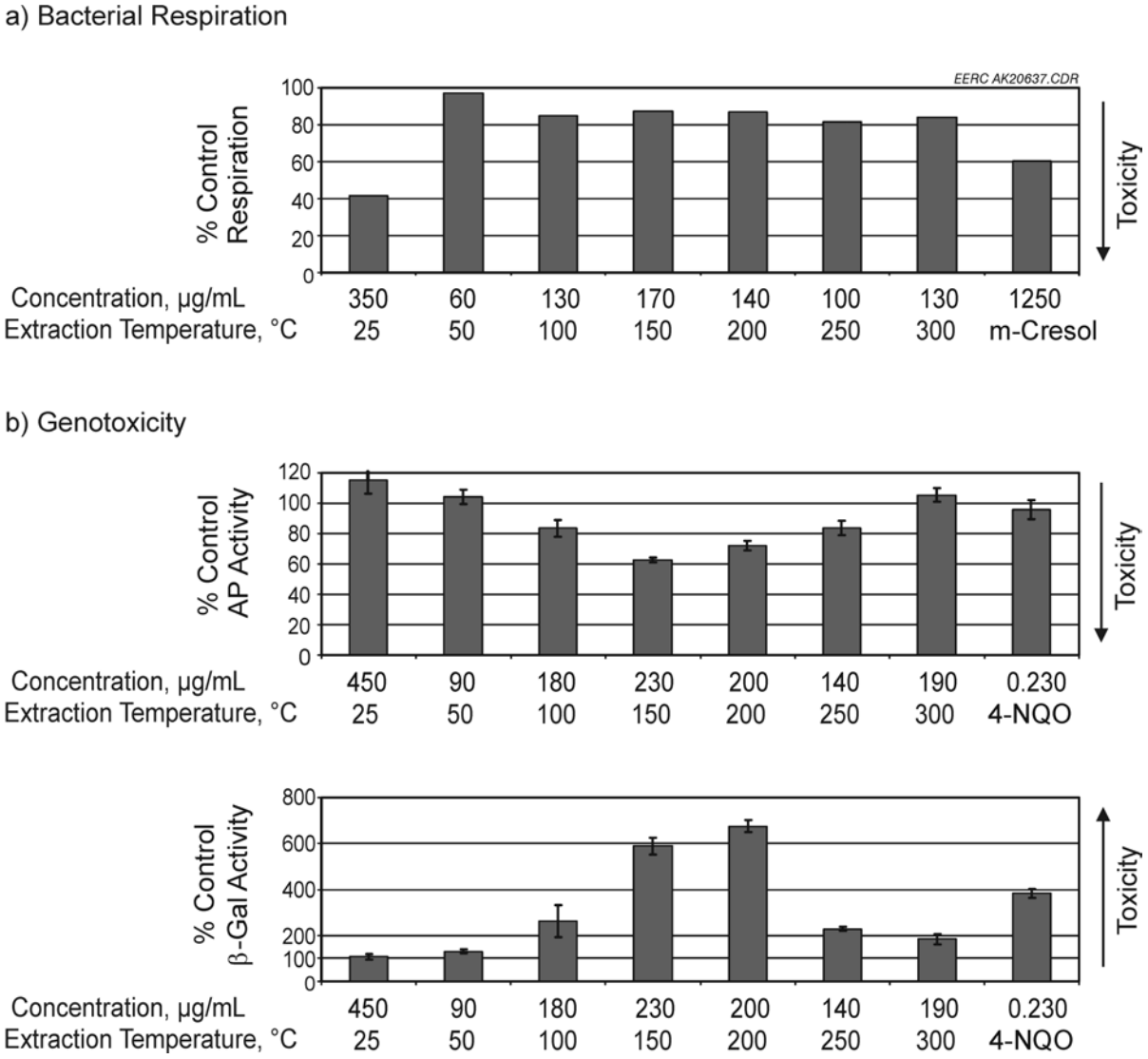

Figure 3. The results from bacterial toxicity tests performed on diesel exhaust particulate fractions obtained sequentially at temperatures 25°–300ºC. Polytox™ 5% of each extract, b) Genotoxicity (0.5%). Each test was validated on test compounds, m-cresol for the Polytox™ and 4-nitroquinoline oxide (NQO) for the genotoxicity test.

be used to approximate the extracted mass of organic compounds, including oxygenated organics, by multiplying the TOC values by a factor estimated to include "average" oxygen content. The most common factor is 1.4, which corresponds mainly to aerosols in urban areas.[26] Recently, it has been shown that for aerosols heavily impacted by wood smoke, a factor of 2.2–2.6 is more suitable.[26] Therefore, for an estimation of total organics in wood smoke and diesel exhaust particulate, which includes oxygenates, we have used an average factor of 2.4 and 1.4, respectively. For most of the wood smoke particulate water fractions, the estimated total organic compound mass corresponds to (or exceeds) the amount extracted. For diesel exhaust, the estimated total organic compound mass is lower than the total

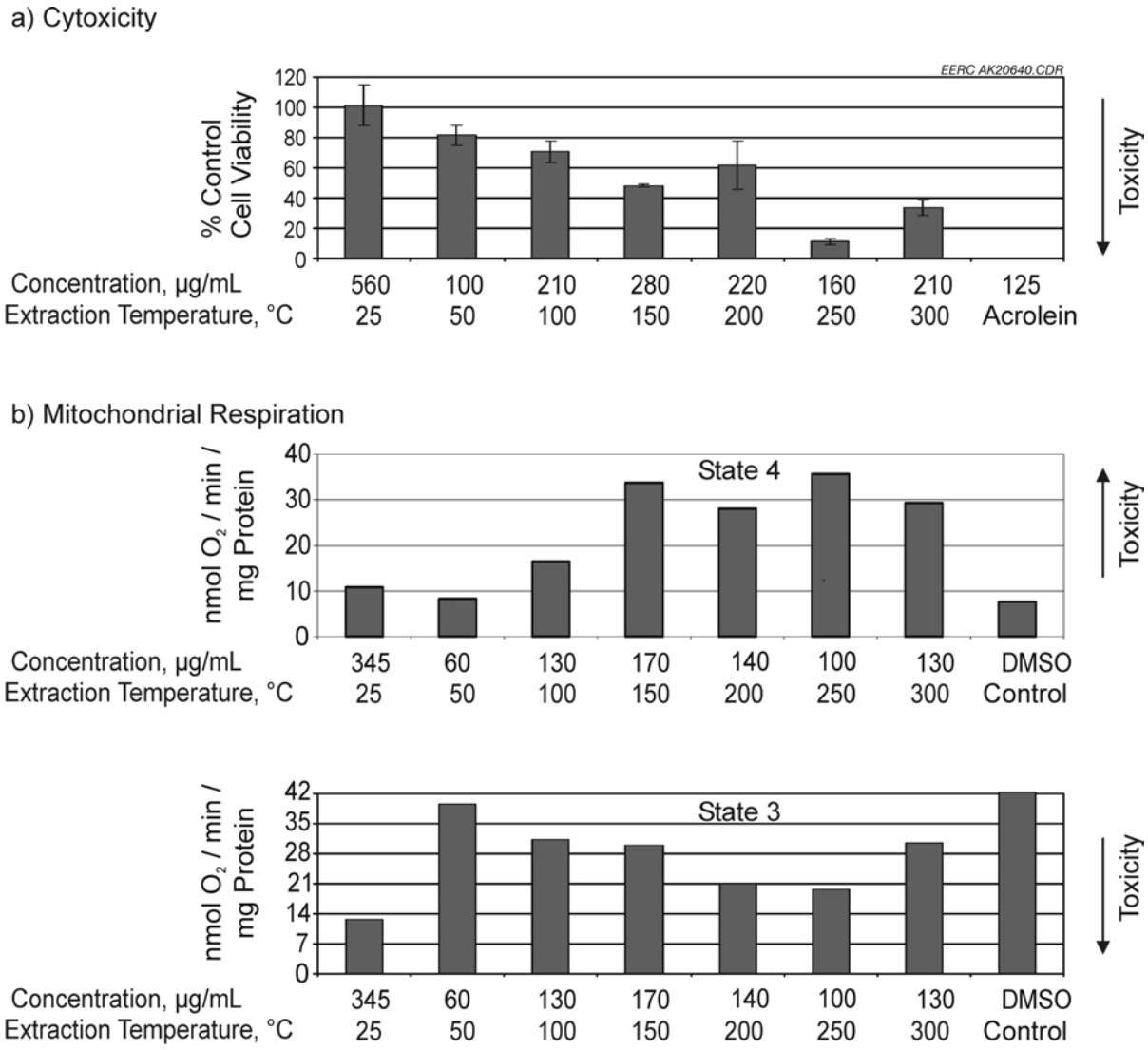

Figure 4. The results from bacterial toxicity tests performed on diesel exhaust particulate fractions obtained sequentially at temperatures 25°–300ºC. a) Cytoxicity 1% of each extract compared to acrolein: b) Mitochondrial respiration (5%) compared to the effect of DMSO.

extracted, probably due to the presence of inorganic compounds, especially in the most polar (25°C) fraction.

The most selective extraction was observed for sulfur (Fig. 5). Increased content of sulfur was found in both samples of the fraction collected at 25°C. The initial concentration of sulfur in diesel exhaust particulate was 40 mg/g. In the lowest-temperature fractions (which were the most toxic), 75% of the sulfur was recovered.

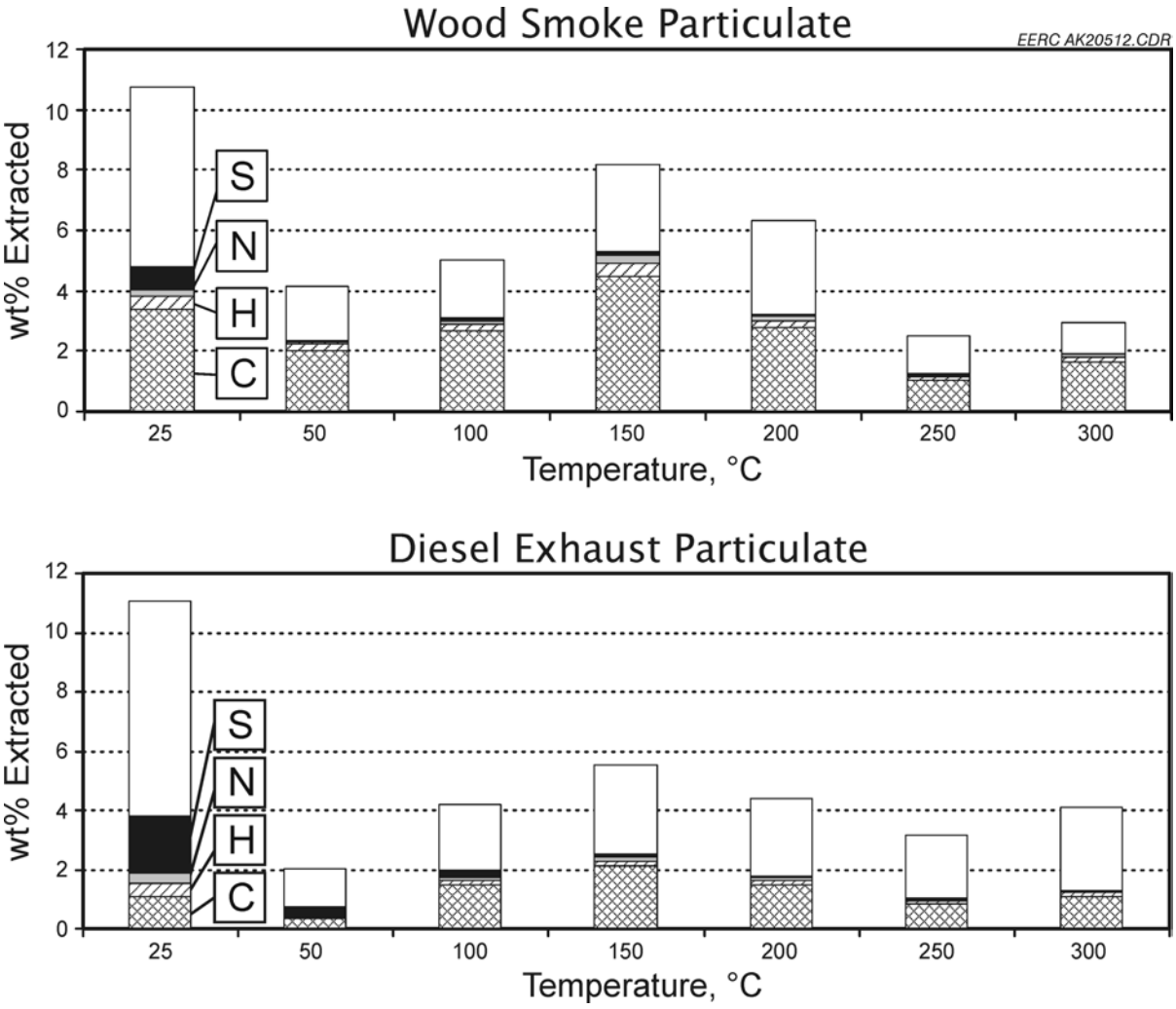

Fig. 5 CHN and sulfur determination on diesel exhaust and wood smoke particulate fractions extracted sequentially with subcritical water at temperatures 25–300°C.

GC/MS and GC/flame ionization detection (FID) analysis

The identification of individual components in each fraction was performed using GC/MS. GC/FID was used for tentative quantification. Although gas chromatography is limited to the determination of mainly nonpolar or slightly polar compounds present in samples, we observed increased concentrations of lignin pyrolysis products such as syringol and quaiacol derivatives, phenols and benzenediols in low-temperature fractions from wood smoke particulate (Fig. 7). Interestingly, a high quantity of levoglucosan, a typical marker of wood burning, was found in water extracts while not observed in acetone extracts. It is important to note that in using GC/MS, we were able to analyze with only ca. 24% of the total extracted mass. GC/MS analysis of diesel exhaust was not as informative as that of wood smoke particulate, because the GC elutable fraction of diesel exhaust consists mainly of alkanes and PAHs. When using GC/MS in selected ion recording mode, selective extraction of

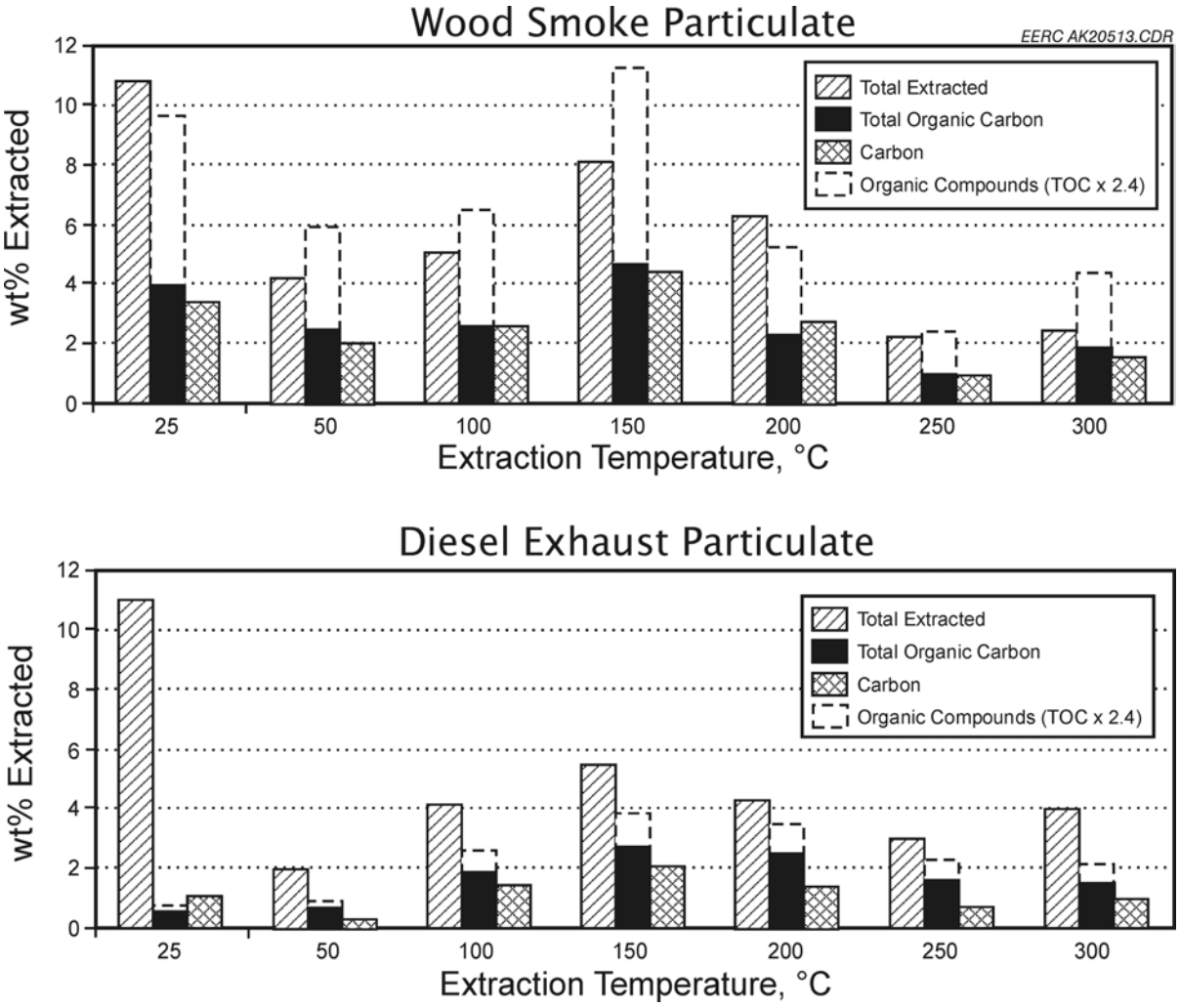

Fig. 6. Determination of extracted mass, total organic carbon, carbon, and estimates of organic compound mass of wood smoke and diesel exhaust particulate fractions extracted sequentially with subcritical water at temperatures 25°–300°C.

nitropyrene at temperature 150° and 200°C was observed. The presence of nitroaromatics would explain genotoxicity of the same diesel exhaust extracts.

Metal analysis

Inductively coupled plasma mass spectrometry (ICP/MS) metal analysis showed high concentrations of zinc, iron, magnesium, and manganese in the most polar fraction (25°C) (Fig. 8). All of these elements were probably present in the form of sulfates, which are soluble in water. The presence of sulfates may correspond to increased concentration of sulfur (Fig. 5) and also to lower pH of 2.5. The increased recoveries of certain metals at different temperatures were probably due to selective extraction of different metal species.

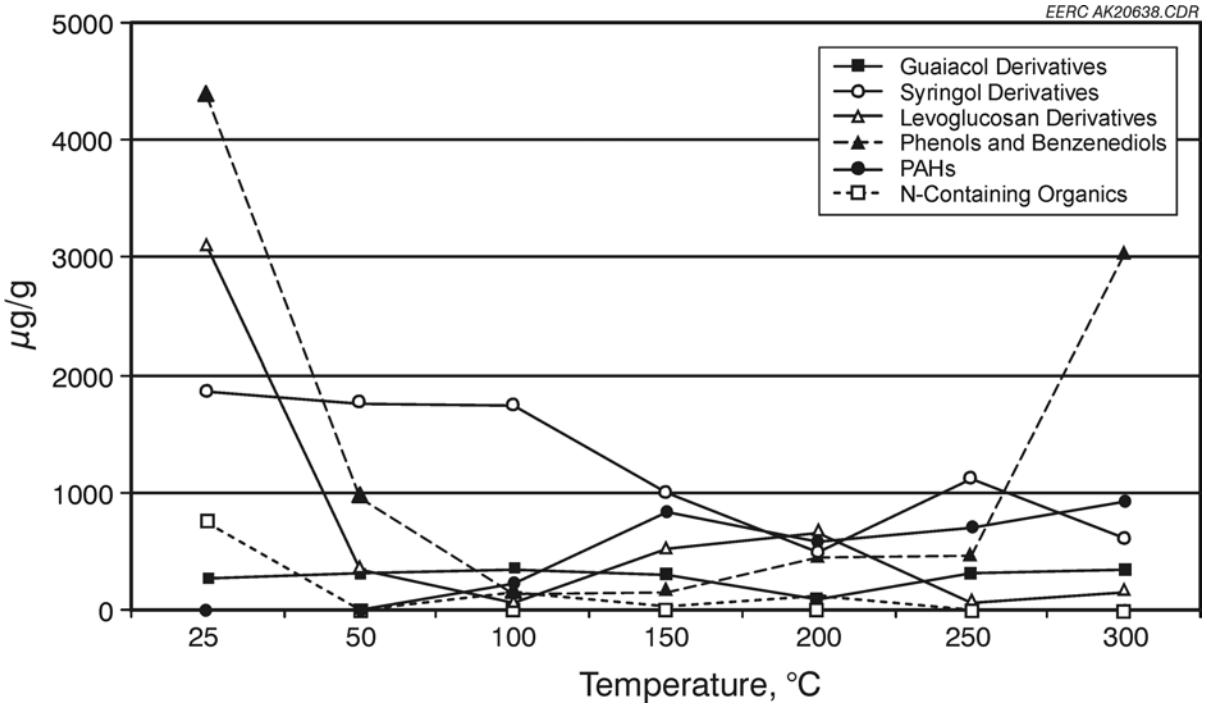

Fig. 7. Concentrations of wood smoke particulate organics in subcritical water extracts collected sequentially at different temperatures.

CONCLUSIONS

- Subcritical water has been shown to selectively extract organic compounds ranging from polar to nonpolar.
- High toxicity was found in the polar fractions, fractions which are not expected to be extracted by organic solvents.
- GC/MS characterization of wood smoke particulate showed phenols, benzenediols, and levoglucosan extracted preferentially in lower-temperature fractions.
- Higher content of sulfur, magnesium, iron, and zinc was found in the lowest-temperature fraction (the most polar) of diesel exhaust particulate.
- The results indicate that standard methods employing organic solvents neglect characterizing the polar fractions of aerosol particulate, which are important from a toxicological point of view.
- Similarly, tests studying leachates (water extracts at ambient temperature) might omit toxicologically important polar fractions extracted above 25°C.
- The initial results show selective response of different toxicity test to each extract. Further testing will study the dependence of toxicity on the mass of extracted particulate.

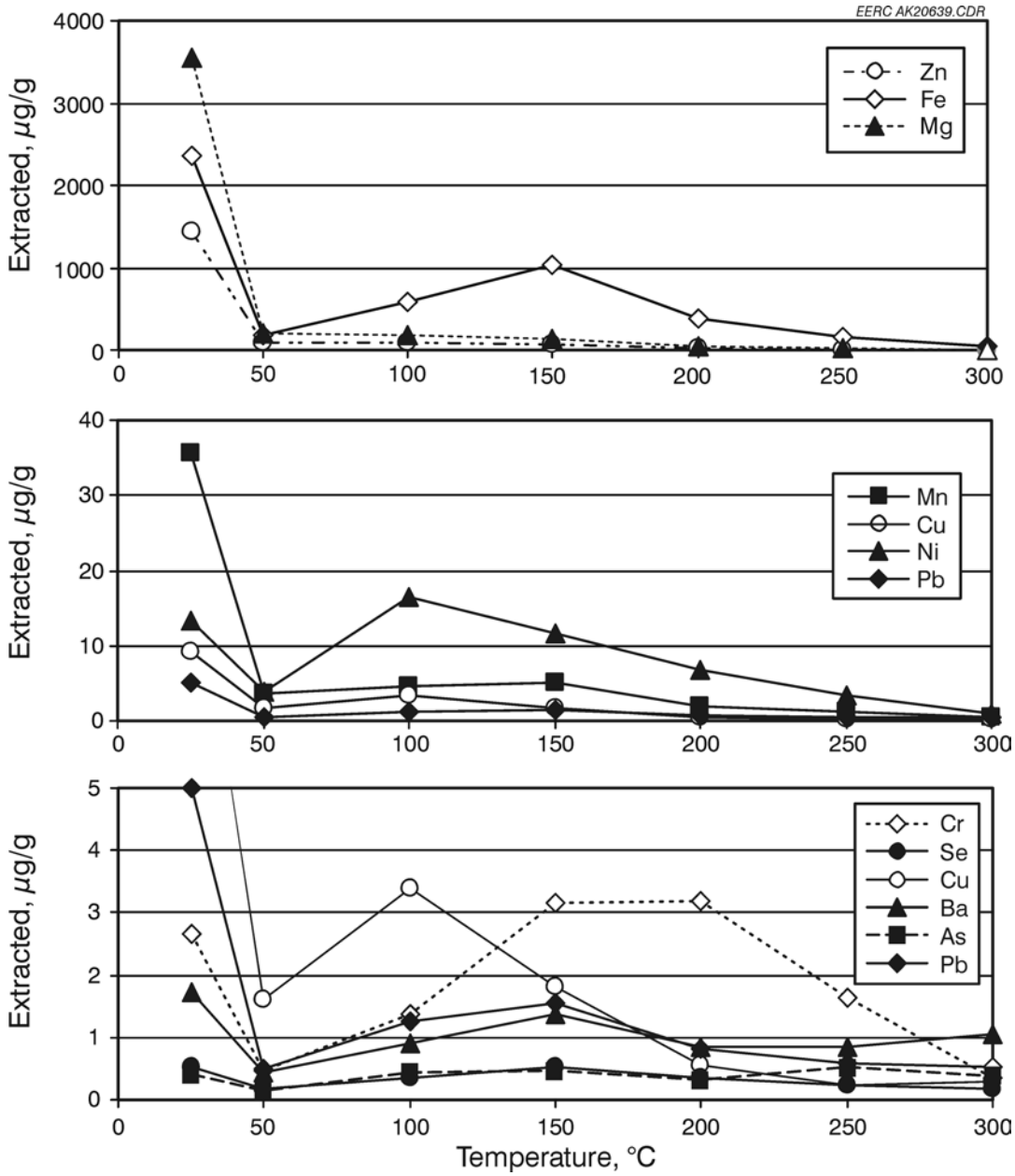

Fig. 8. Determination of metals in diesel exhaust particulate using ICP/MS.

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REFERENCES

1. Hannigan, M.P.; Cass, G.R.; Penman, B.W.; Crespi, C.L.; Lafleur, A.L.; Busby, W.F. Jr.; Thilly, W.G. *Environ. Sci. Technol*. **1997**, 31, 438.
2. Reichhardt, T. *Environ. Sci. Technol*. **1996**, 30, 68A.
3. Christoforou, C.C.; Salmon, L.G.; Hannigan, M.P.; Cass, G.R. *Air & Waste Manage. Assoc*. **2000**, 50, 43.
4. Benson, S. EERC. Personal communication, October, 2000.
5. Hawthorne, S.B.; Miller, D.J.; Lagadec A.J.M. U.S. Patent 6, 001, 256, 2000.
6. Hawthorne, S.B.; Miller, D.J.; Lagadec, A.J.M.; Hammond, P.J.; Clifford, A.A. U.S. Patent 6, 352, 644, 2002.
7. Yang, Y.; Hawthorne, S.B.; Miller, D.J. *Environ. Sci. Technol*. **1997**, 31, 430.
8. Moolgavkar, S.H.; Luebeck E.G. *Epidemiology* **1996**, 7, 420.
9. Squadrito, G.L.; Cueto, R.; Dellinger, B.; Pryor, W.A. *Free Rad. Biol. & Med*. **2001**, 31, 9.
10 Bitton, G.; Koopman, B. *Rev. Environ. Contam. Toxicol.* **1992**, 125,1.
11. Oanh, N.T.K.; Nghiem, L.H.; Phyu, Y.L. *Environ. Sci. Technol*. **2002,** 36, 833.
12. Buschini, A; Cassoni, F.; Anceschi, E.; Pasini, L.; Poli, P.; Rosi C. *Chemosphere* **2001**, 44, 1723.
13. Li, N.; Venkatesan, M.I.; Miguel, A.; Kaplan, R.; Gujuluva, C.; Alam, J.; Nel, A. *J. Immunol.* **2000**, 165, 3393.
14. Hawthorne S.B.; Miller, D.J.; Barkley, R.M.; Krieger, M.S. *Environ. Sci. Technol.* **1988**, 22, 1191.
15. Miller, D.J.; Hawthorne S.B. *Anal. Chem.* **1998**, 70, 1618.
16. Franson, M.A.H., Ed. *Standard methods for the examination of water and wastewater.* Am. Public Health Assoc. **1989**, Part 5210B.
17 Plumb, J.A.; Milroy, R.; Kaye, S. *Cancer Res*. **1989**, 49, 4435.
18. Mosmann, T. *J. Immunol. Methods.* **1983**, *65*, 55.
19. Quillardet, P.; Huisman, O.; Ari, R.D.; Hofnung, M. *Proc. Nat. Acad. Sci*. **1982,** 79, 5971.
20. Maron, D.M.; Ames, B.N. *Mut. Res*.**1983**, 113,173.
21. Quillardet, P.; Hofnung, M. *Mut.Res*. **1985**,147, 65.
22. Mersch-Sundermann, V.; Kevekordes, S.; Mochaydei, *S. Mut. Res*. **1991**, 252, 51.
23. Miller, J.H. Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for Escherichia Coli and Related Bacteria. Cold Spring Harbor Laboratory Press, 1992, CSHL Press, USA.
24. Mersch-Sundermann, V.; Wintermann, F.; Kern, S.; Hof, H. 1993 *Mut. Res*. **1993**, 291, 53.
25. Picklo, M.J.; Montine, T.J. *Biochem. Biophys. Acta* **2001**, *1535*, 145.
26. Turpin, B.J.; Saxena, P.; Andrews, E. *Atm. Environ.* **2000**, *34*, 2983.